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Short report

Modification of IgH PCR clonal analysis by the addition of sucrose and cresol red directly to PCR reaction mixes

E Hodges, S M Boddy, S Thomas, J L Smith

Abstract

Diagnostic immunoglobulin (Ig) polymerase chain reaction (PCR) clonality analyses need to be simple, reproducible, and rapid. Sucrose and cresol red (gel loading buffer reagents) were added to a routine IgH PCR reaction mix to obviate the need for adding gel loading buffer separately after PCR amplification. Not only did this decrease the time spent after PCR analysis but also gave similar or enhanced IgH PCR product intensity compared with normal IgH PCR conditions on polyacrylamide gel electrophoresis. This procedure was easily adapted to routine PCR analysis without the need for further manipulations or optimisation of the PCR reaction mix, and it increased the reproducibility and specificity of the IgH PCR products.

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Keywords: immunoglobulin; polymerase chain reaction; sucrose; cresol red

Routine polymerase chain reaction (PCR) clonality analyses of lymphoproliferative diseases need to be simple, reproducible, rapid, and cost effective. Our routine IgH PCR methods use a limited number of primers that result in PCR products of 80–150 base pairs (FR3/JH) and 240–280 base pairs (FR2/JH) that can be run on high density polyacrylamide gels.[1] We describe the addition of sucrose and cresol red directly to the reaction mixes before IgH PCR amplification to decrease time spent on post-PCR manipulations.[2] This method also increased the specificity and intensity of the IgH PCR products and was applicable to DNA extracted from formalin fixed paraffin wax embedded tissue.

Methods

Thirteen cases of histologically and immunophenotypically defined B cell lymphoproliferative diseases—two cases of B cell lymphocytic leukaemia, one case of hairy cell leukaemia, and 10 cases of B cell non-Hodgkin's lymphoma—were entered into the study, as well as three normal healthy controls.

DNA was extracted from fresh EDTA peripheral blood samples and thawed lymph node cells by a rapid salting out technique, and from paraffin wax embedded samples by a routine method involving xylene washes and proteinase K incubation as described previously.[1]

IgH CDR3 PCR analysis was performed using FR3 and JH primers essentially as described by Potter *et al.*[3] The PCR reaction mix consisted of 1 μg DNA, 250 ng of each primer, 200 μM each dNTP and 1 unit Taq polymerase (Amersham International, Buckinghamshire, UK). The reactions were subjected to 30 cycles of PCR (93°C for one minute, 55°C for one minute, 72°C for one minute). PCR products were electrophoresed on a 10% polyacrylamide gel and stained with ethidium bromide. The sizes of IgH FR3 products range from 60–150 base pairs. The IgH FR2 PCR analysis was performed in a semi-nested PCR amplification using primers FR2/LJH and FR2/VLJH as described by Diss *et al.*[4] The first round PCR reaction mix consisted of 250 ng DNA, 20 pmol of each primer, 200 μM each dNTP, and 1 unit Taq polymerase. The second round PCR reaction consisted of 0.5 μl of the primary PCR products and 0.5 units Taq polymerase. Samples were subjected to 30 first round and 20 second round cycles of PCR (93°C for 45 seconds, 50°C for 45 seconds, 72°C for 110 seconds). PCR products were electrophoresed on a 5% polyacrylamide gel and stained with ethidium bromide. Sizes of the IgH FR2 PCR products range from 240–280 base pairs.

In addition to all IgH FR3 PCR reaction mixes and IgH FR2 second round PCR reaction mixes, sucrose and cresol red were added directly to the PCR reaction mixes. For optimising conditions, sucrose and cresol red were added at varying final concentrations from 3–25% and 0.05–0.4 mM, respectively, using a stock solution of 60% sucrose and 1 mM cresol red; and for initial experiments using known positive, negative, and polyclonal controls. After the PCR amplifications had been completed, PCR products were loaded directly onto the polyacrylamide gels without

Molecular Immunology Group, Wessex Immunology Service, Southampton University Hospitals NHS Trust, Tremona Road, Southampton SO16 6YD, United Kingdom
E Hodges
S M Boddy
S Thomas
J L Smith

Correspondence to:
Dr E Hodges.

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Table 1 Comparison of IgH PCR products obtained from normal PCR conditions with those obtained by adding sucrose and cresol red directly to the PCR reaction mix

			IgH FR3				*IgH FR2*			
Case	*Diagnosis*	*Tissue*	*Normal conditions*		*Sucrose/cresol red*		*Normal conditions*		*Sucrose/cresol red*	
1	B CLL	PB	C	+++	C	+++	C	+++	C	+++
2	B CLL	PB	C	++	C	+++	ND		ND	
3	HCL	PB	C	+++	C	+++	C	+++	C	+++
4	B NHL	Frozen LN	N		P	++	C	++	C	+++
5	B NHL	Frozen LN	C	+++	C	+++	C	+++	C	+++
6	B NHL	Frozen LN	C	++	C	++	ND		ND	
7	B NHL	Paraffin LN	C	++	C	++	ND		ND	
8	B NHL	Paraffin LN	N		C	+	ND		ND	
9	B NHL	Paraffin LN	N		C	(+)	C	++	C	++
10	B NHL	Paraffin LN	P	+++	P	+++	C	+++	C	+++
11	B NHL	Paraffin LN	C	++	Cø	+	C	+++	C	+++
12	B NHL	Paraffin LN	C	+	C	+	P	++	P	++
13	B NHL	Paraffin LN	C	+	C	++	O	++	C(P)	++
14	Control	PB	P	++	P	++	P	++	P	++
15	Control	PB	P	++	P	++	P	++	P	++
16	Control	PB	P	++	P	++	P	++	P	++

Intensity of clonal/polyclonal PCR products: +++, strong; ++, medium; + weak; (+) faint.
Cø, the clonal band was marginally less intense in the sucrose/cresol red reaction than under normal conditions.
B CLL, B cell lymphocytic leukaemia; HCL, hairy cell leukaemia; B NHL, B cell non-Hodgkin's lymphoma; PB, peripheral blood; LN, lymph node; C, clonal; P, polyclonal; N, negative; O, oligoclonal; C(P), clonal band in polyclonal smear; ND not done.

further manipulations. Dilution experiments indicated that a final concentration of 12% sucrose and 0.2 mM cresol red was optimum for both IgH PCR protocols (data not shown).

Results

Thirteen cases of B cell lymphoproliferative diseases and three normal healthy controls were analysed by IgH clonality PCR techniques with the addition of 12% sucrose and 0.2 M cresol red directly to the PCR reaction mix before amplification. Results were correlated directly with those obtained under normal PCR conditions—that is, without sucrose or cresol red, and scored blindly by three independent observers (table 1, fig 1) on the clonal/polyclonal nature of the product and intensity/definition of PCR product.

In three normal healthy controls the polyclonal PCR products were comparative in both sets of reactions with no obvious visual differences for both IgH FR3 and FR2 PCR.

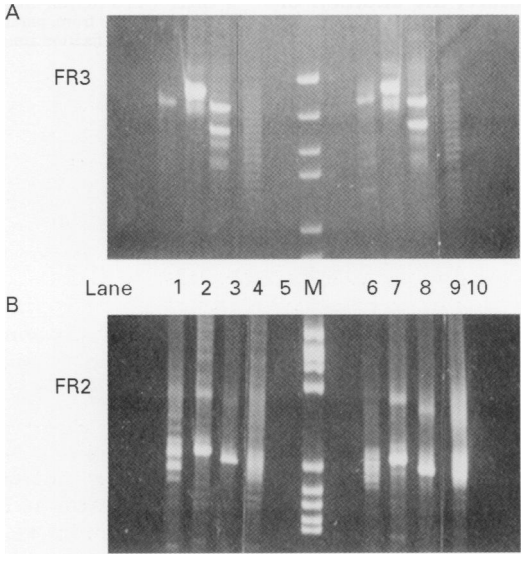

Figure 1 (A) IgH FR3 and (B) IgH FR2 PCR analysis. PCR products from normal PCR conditions are shown in lanes 1–5 and comparative PCR products in which sucrose and cresol red were added directly to the PCR reaction mix are shown in lanes 6–10. DNA molecular weight marker pBR322 HaeIII is shown in lane M; case 13 in lanes 1 and 6; case 1 in lanes 2 and 7; case 3 in lanes 3 and 8; polyclonal DNA control in lanes 4 and 9; and negative control in lanes 5 and 10.

In two of six cases of B cell neoplasia (cases 2 and 4) in which DNA was isolated from fresh or frozen material, the addition of sucrose and cresol red improved the resultant IgH PCR product as judged by polyacrylamide gel electrophoresis. In the remaining four cases (cases 1, 3, 5, and 6) IgH PCR products were viewed to be of similar intensity and definition in both sets of reactions.

In three of seven cases of B cell non-Hodgkin's lymphoma (cases 8, 9, and 13), in which DNA was isolated from paraffin wax embedded biopsies, the addition of sucrose and cresol red increased the intensity and definition of the PCR products compared to the PCR products obtained under normal condition for IgH FR3 PCR. In two of these cases (cases 8 and 9) a faint clonal product was seen only after the addition of sucrose and cresol red. Furthermore, in case 13, a more definitive clonal band on a polyclonal background was also observed for IgH FR2 PCR whereas an indistinct oligoclonal pattern was observed using usual PCR conditions. Of the remaining four cases, three (cases 7, 10, and 12) were judged to have similar PCR products for IgH FR3 or IgH FR2 or both, and in one case (case 11) normal PCR conditions gave a slightly more intense PCR product for IgH FR3.

The addition of sucrose and cresol red did not induce any false negative or false positive PCR reactions in the PCR controls.

Discussion

We investigated the addition of gel loading buffer reagents, sucrose and cresol red, directly to PCR reaction mixes before PCR amplification for IgH clonality studies. This negates the need for adding gel loading buffer separately to each PCR product after PCR amplification but before gel electrophoresis. Sucrose and cresol red were used as alternatives for the components of conventional gel loading buffer, Ficoll and bromophenol blue, which inhibit PCR amplification.[2] The inclusion of sucrose has already been reported to increase the efficiency

of HLA class II PCR-SSP amplification and is routinely used in HLA typing.[5 6]

Initial titration experiments indicated that a final concentrations of 12% sucrose and 0.2 mM cresol red were optimum for use in the FR3/JH PCR reaction mix and in the second round FR2/VLJH reaction mix. DNA isolated from 13 B cell neoplasms and three normal healthy controls was entered into the comparative study (table 1, fig 1).

In five cases the IgH PCR product was viewed to be more intense and more definite with the addition of sucrose and cresol red to the PCR reaction mix than under normal PCR conditions. These included cases in which DNA was extracted from either frozen or paraffin wax embedded specimens. The increase in PCR product may result from a sucrose induced increase in reaction mixture osmolarity; therefore, it may be of value to investigate other methods of increasing osmolarity to examine any change in PCR specificity. In two of these cases an IgH FR3 PCR product was clearly visible with the addition of sucrose and cresol red that was undetectable under normal conditions. Interestingly, in both these cases, DNA had been isolated from paraffin wax embedded tissue. It is feasible therefore that these modifications may overcome problems incurred when amplifying DNA extracted from formalin fixed, paraffin wax embedded biopsies because of the presence of inhibitors from fixation and processing procedures.[7 8] In seven cases, IgH PCR products from both normal PCR conditions and sucrose and cresol red PCR conditions gave similar PCR products. In only one case was the PCR product assessed to be marginally stronger under normal PCR conditions than under the modified conditions. However in this case a clonal product was identified from both sets of reactions and therefore the clonal nature of the specimen was never in doubt. Furthermore, the addition of sucrose and cresol red did not induce any false positive or false negative results in the PCR controls. These results indicate that the addition of sucrose and cresol red is amenable to PCR reactions regardless of the source of the DNA.

This simple modification to the IgH PCR protocol not only induced strong specific PCR bands in some cases but also negated the use of any post-PCR manipulations, thereby decreasing the possibility of contamination and reducing manual time spent loading PCR reactions onto polyacrylamide gels. Furthermore, the high density of the sucrose containing samples made them easier to load and the cresol red eliminated the dark shadows occasionally seen with ethidium bromide stained gels with traditional blue dyes. It also reduced consumable costs associated with post-PCR manipulations.

This is a very efficient modification to standard IgH PCR protocols and we are now optimising the concentrations of sucrose and cresol red for use with our routine T cell receptor (TCR)B and TCRG PCR protocols.

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